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The role of hypoxia and HIF1α in the regulation of STAR-mediated steroidogenesis in granulosa cells

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ABSTRACT

The adaptive responses to hypoxia are mediated by hypoxia-inducible factor 1 alpha (HIF1α). Its role, however, in regulating steroidogenesis remains poorly understood. We examined the role of hypoxia and HIF1α in regulating steroid acute regulatory protein (STAR) expression and steroidogenesis in immortalized (KK1) mouse granulosa cells under progressively lowering O_2 concentrations (20%, 15%, 10%, 5%, 1%). Basal and dbcAMP-stimulated progesterone synthesis was decreased under severe hypoxia (1% and 5% O_2). The partial hypoxia revealed opposing effects, with a significant increase in steroidogenic response at 10% O_2 in dbcAMP-treated cells: *Star*-promoter activity, mRNA and protein expression were increased. The hypoxia-stimulated STAR expression was PKA-dependent. Binding of HIF1α to the *Star*-promoter was potentiated under partial hypoxia. Inhibition of the transcriptional activity or expression of HIF1α suppressed STAR-expression. HIF1α appears to be a positive regulator of basal and stimulated STAR-expression, which under partial hypoxia is capable of increasing the steroidogenic capacity of granulosa cells.

1. Introduction

Within the ovary, growing oocytes together with accompanying granulosa cells (*i.e.*, the cumulus–oocyte complex) are surrounded by follicle structures, the vascular supply of which is restricted to the *theca interna* layers (Grazul-Bilska et al., 2007; Martelli et al., 2009; Suzuki et al., 1998; Zeleznik et al., 1981). The follicular basement membrane constitutes the borderline between the vascularized and non-vascularized follicle segments. Following ovulation, spreading blood vessels cross the basement membrane and establish the luteal vascular network, which in mature corpus luteum provides virtually every lutein cell with access to a capillary. Consequently, oocyte growth, maturation and ovulation, as well as the subsequent early development of the corpus luteum, processes associated with increased steroidogenic activity, take place under reduced oxygen tension (Fischer et al., 1992; Fraser et al., 1973; Huey et al., 1999; Imoedemhe et al., 1993; Redding et al., 2008; Shalgi et al., 1972). Thus, hypoxia appears to be an integral part of the ovarian environment during follicle maturation. One of the most prominent factors involved in the physiological responses to hypoxia is the hypoxia-inducible factor 1 alpha (HIF1α). Under hypoxic conditions, HIF1α, which is normally rapidly degraded by the ubiquitin–proteasome pathway, becomes stabilized and translocates to the nucleus where, upon dimerization with its constitutively-expressed HIF1β subunit, binds to the hypoxia response element (HRE) of target genes, initiating their expression (Semenza, 1998). Besides hypoxia, among other factors inducing HIF1α expression under normoxic conditions are inflammatory mediators such as, *e.g.*, prostaglandins (Fukuda et al., 2003), interferons (Gerber and Pober, 2008) or growth factors (Herr et al., 2004).

Studies aiming to delineate the progesterone receptor (PGR)-dependent pathways involved in ovulation identified HIF1α as one of the downstream mediators of PGR function during the ovulatory period (Kim et al., 2009). Following this observation, in the same study it was shown that blocking HIF1α transcriptional activity by echinomycin, a compound inhibiting HIF1α DNA binding capabilities and, hence, its transcriptional activity, inhibits the expression of PGR-regulated genes that are critical for ovulation, resulting in impaired ovulation (Kim et al., 2009). Furthermore, increased HIF1α expression was observed in ovaries from hCG-stimulated mice (Kim et al., 2009). Similarly, in another study, a synergy between effects of low oxygen tension (partial hypoxia) and hCG, resulting in upregulation of HIF1α activity, was observed in luteinizing mouse granulosa cells both *in vitro* and *in vivo* (Tam et al., 2010). HIF1α was maximally induced during follicle maturation, especially in granulosa cells around ovulation. This additive effect of hCG on HIF1α expression was present in luteinizing granulosa cells when HIF1α

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was stabilized by cobalt chloride ($CoCl_2$) (Tam et al., 2010). Moreover, HIF1α is one of the master regulators in the expression of vascular endothelial growth factor A (VEGF), an angiogenic and luteotropic factor contributing to luteal formation (Zhang et al., 2011).

Thus, cumulatively, there is strong evidence suggesting an essential role of hypoxia-regulated factors, and especially of HIF1α, during follicular and luteal development (Zhang et al., 2011). Strongly contrasting with this idea are data disputing the involvement of hypoxia in ovarian steroidogenesis, mostly reporting adverse instead of stimulatory effects (Jiang et al., 2011; Nishimura et al., 2006). These data were, however, primarily related to severely hypoxic conditions and reflected an attenuated expression and/or function of steroidogenic acute regulatory (STAR) protein regulating acute steroid production, and of the CYP11A1 (P450scc) enzyme, which is responsible for conversion of cholesterol to pregnenolone (Jiang et al., 2011; Nishimura et al., 2006). Thus, it appears that the degree

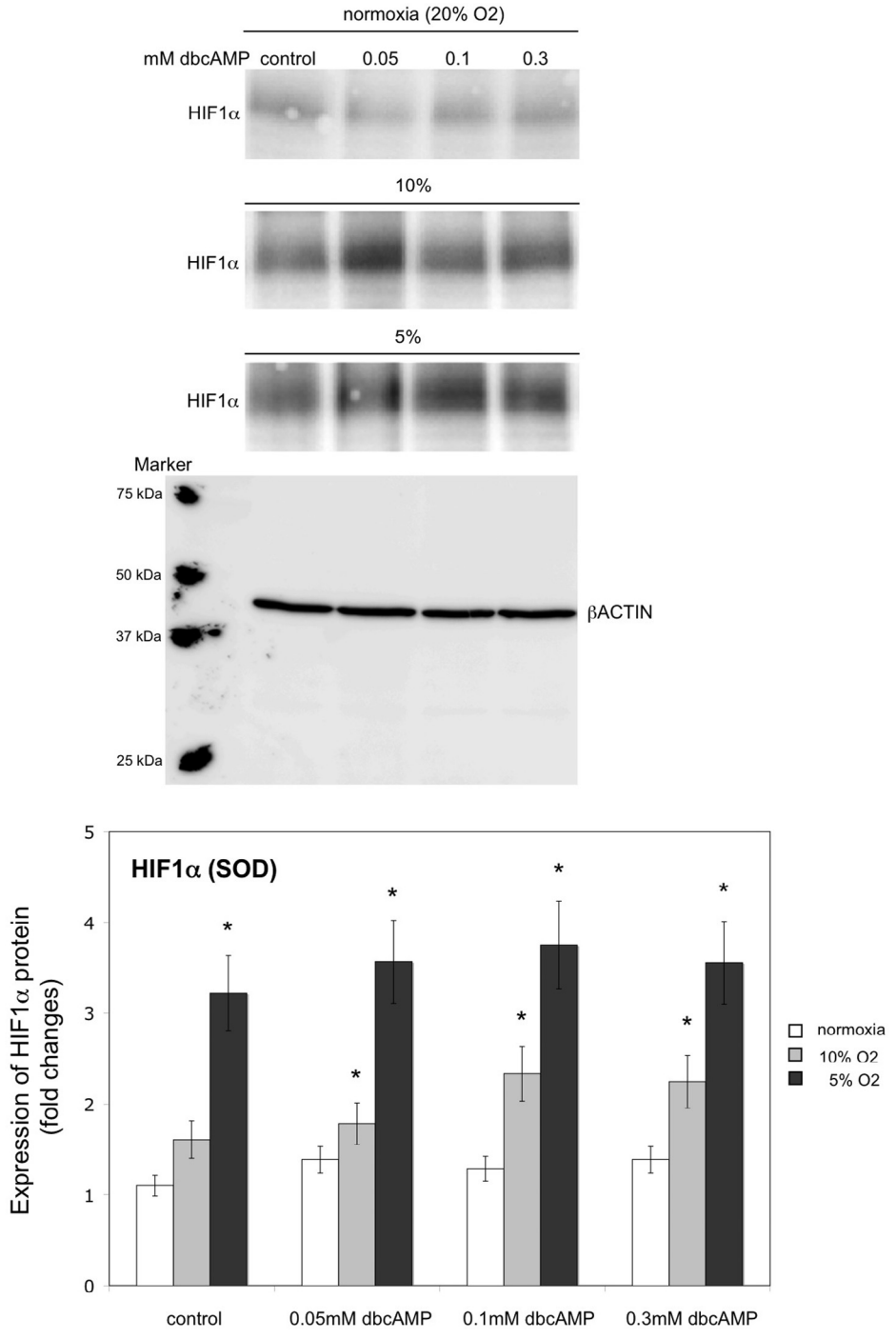

Fig. 1. Expression of HIF1α under normoxia (20% oxygen, O_2) and decreased O_2 concentrations (10% and 5%) in immortalized KK1 mouse granulosa cells. Cells were cultured in DMEM/F12 medium with increasing dbcAMP concentrations and different O_2 concentrations for 6 h. Cells were collected, homogenized and 20 μg of the lysate was used for Western blot analysis of HIF1α (120 kDa) and βACTIN (45 kDa). One-way ANOVA ($P < 0.0001$) followed by Dunnett's multiple comparison test was applied; the average standardized optical density (SOD) for HIF1α is presented as *n*-fold changes relative to untreated normoxic (20% O_2) control; (*) indicates $P < 0.01$. Representative Western blots are shown. An exemplary resolution of the Precision Plus Protein Standard (marker) annotated using the phosphorescent ink marking pen (as described in materials and methods) is presented on membrane reblotted for βACTIN.

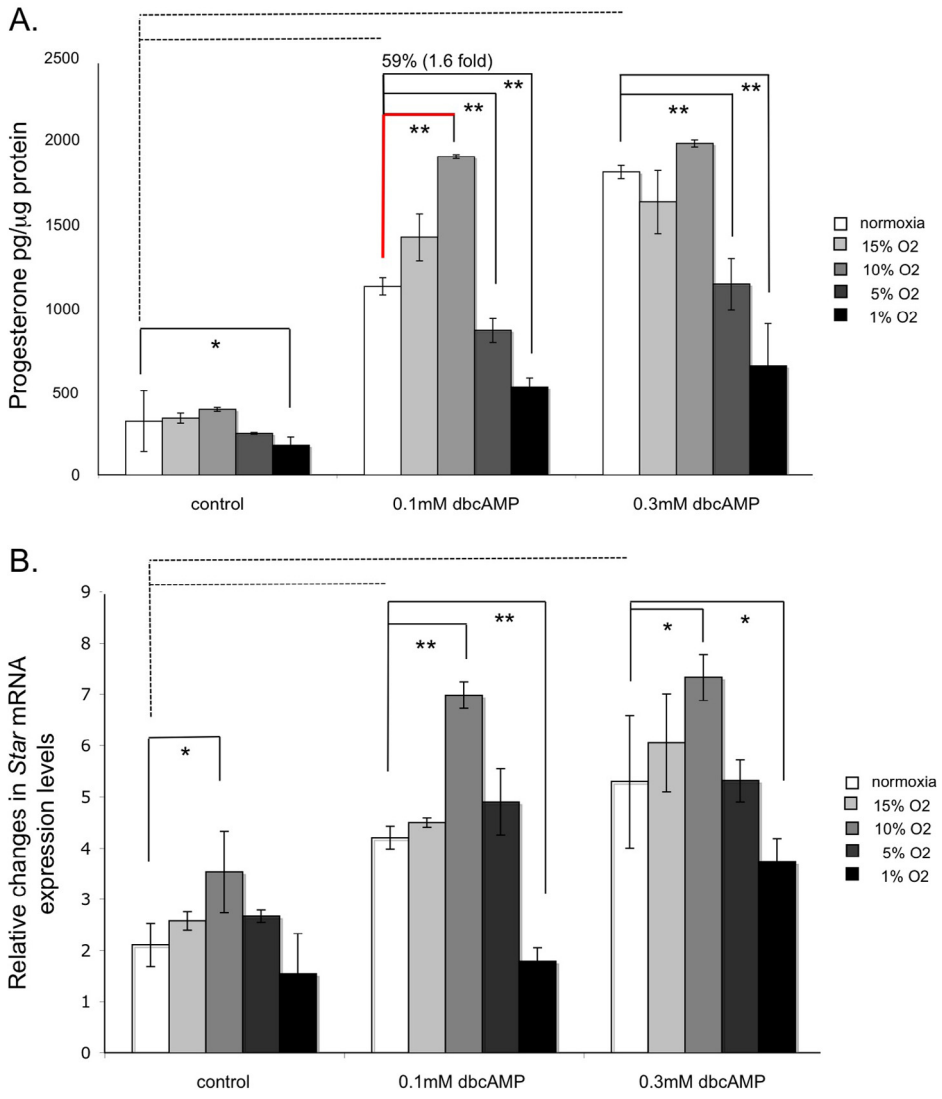

Fig. 2. Steroidogenesis and expression of *Star* mRNA in KK1 cells cultured under progressively decreasing O_2 concentrations in the presence of the background PKA activity. Cells were treated for 6 h in DMEM/F12 medium without or with 0.1 mM or 0.3 mM dbcAMP at different O_2 concentrations. (A) The culture medium was collected and progesterone production was assessed by radioimmunoassay. One-way ANOVA with $P < 0.0001$ for each of the groups, followed by Dunnett's multiple comparison test was applied; all samples were compared against the normoxic control in each group. (*) indicates $P < 0.05$ and (**) indicates $P < 0.01$. (B) *Star* mRNA expression was determined by Real Time (TaqMan) PCR. One-way ANOVA (ANOVA $P < 0.01$ for control, $P < 0.0001$ for 0.01 mM dbcAMP and $P < 0.004$ for 0.3 mM dbcAMP- stimulated cells), followed by Dunnett's multiple comparison test was applied; all samples were compared against the normoxic control in each group. (*) indicates $P < 0.05$ and (**) indicates $P < 0.01$. Additionally, one-way ANOVA followed by Dunnett's Multiple Comparison Test was applied for all controls in (A) and (B) (dashed lines indicate $P < 0.01$).

of hypoxia is an important factor in regulating steroidogenesis. It has also been demonstrated that severe hypoxia inhibited the phosphorylation of protein kinase A (PKA) (Jiang et al., 2011). The cAMP/PKA pathway is the major signaling cascade regulating STAR expression and function (Dyson et al., 2009; Kowalewski et al., 2010; Manna et al., 2002). It thus appears plausible that inhibition of PKA phosphorylation could be one of the factors inhibiting steroidogenesis under severely hypoxic condition.

Therefore, it appears to be important to distinguish between mild (partial or physiological) and severe hypoxia initiating adaptive responses in affected tissues. This has been clearly shown in studies concerning the role of hypoxia in apoptosis (Greijer and van der Wall, 2004; Piret et al., 2002): whereas severe hypoxia may lead to apoptosis, partial hypoxia can actually be beneficial for cells or organs, resulting in anti-apoptotic effects and/or increased proliferation rates.

This oxygen tension-dependent expression and function of HIF1α could also account for its effects on steroidogenic properties of follicle-enclosed granulosa cells, especially since the presence of HIF1α is mandatory for proper ovarian function (Kim et al., 2009). In view of these observations, presently, the role of hypoxia and HIF1α in regulating steroidogenesis, and specifically in regulating STAR protein expression, needs to be considered as not well understood. Therefore, in order to obtain more detailed insights into the underlying regulatory mechanisms, the impact of progressively reduced oxygen levels on both basal and stimulated STAR-mediated steroidogenesis was examined in immortalized mouse granulosa (KK1) cells used as a cell culture model.

2. Materials and methods

2.1. Cell cultures, transfections and luciferase assay

Immortalized KK1 mouse granulosa cells (kindly provided by Dr. Ilpo Huhtaniemi, Hammersmith Campus, Imperial College, London, UK) were used for all experiments and were cultured in DMEM/F12 medium containing 10% heat-inactivated fetal bovine serum (FBS),

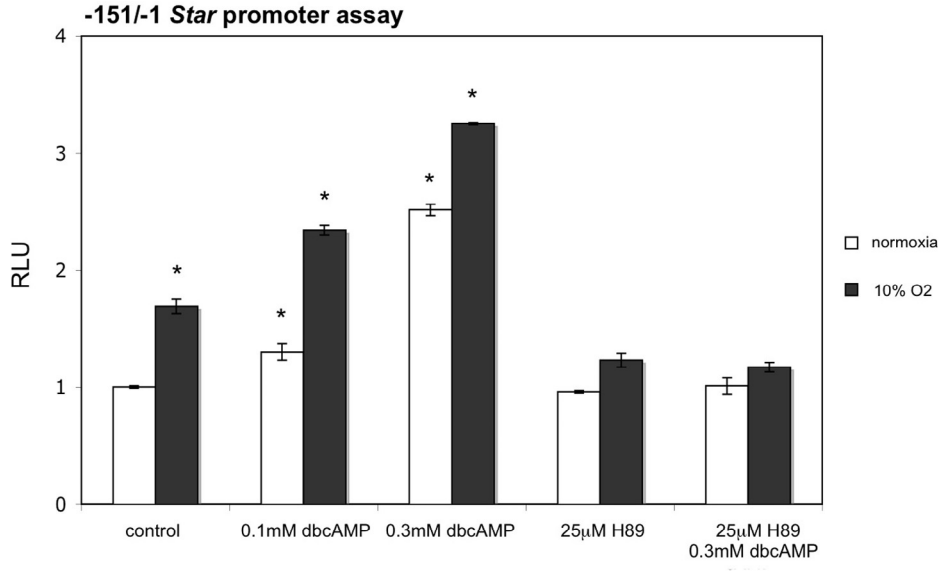

Fig. 3. Partial (10% O_2) hypoxia- mediated increase of *Star* promoter activity is prevented by blocking protein kinase A (PKA) activity in KK1 mouse granulosa cells. Cells were transfected with a -151/-1 bp fragment of *Star* promoter subcloned to pGL3 vector containing Firefly luciferase as a reporter. Transfection efficiency was normalized by cotransfecting pRL-SV40 vector constitutively expressing *Renilla* luciferase. Cells were treated for 6 h. Treatment with PKA blocker (20 μM H89) prevented the stimulation of STAR expression both under normoxic and partial hypoxic (10% O_2) conditions. One-way ANOVA with $P < 0.0001$ followed by Dunnett's multiple comparison test was applied; all samples were compared with the untreated normoxic control; (*) indicates $P < 0.05$. RLU = relative light units.

100 U/ml penicillin and 100 ml/ml streptomycin (all purchased from Chemie Brunschwig AG, Basel, CH) in a humidified incubator at 37 °C and 5% CO_2 in air. The KK1 cell line was developed using transgenic mice expressing SV40 T-antigen driven by the fragments of the inhibin-α subunit promoter (Kananen et al., 1995). Initially the cells displayed LH and FSH receptor responsiveness (Kananen et al., 1995). They express steroidogenic enzymes and exhibit a dose-dependent steroidogenic response when stimulated with N6,2-dibutyryladenosine-3,5-cyclic monophosphate (dbcAMP). Twenty-four hours before experiments, cells were seeded on 6-well plates allowing them to be used for experiments at 60–70% confluence on the next day. Following this, cells were preincubated under respective experimental conditions for 24 h before stimulation. The serum-containing culture medium was then removed and, following washing with phosphate buffered saline (PBS) (Chemie Brunschwig AG), replaced by serum-free medium that was preconditioned for 24 hours before stimulation experiments. Based on our previous experiments (Kowalewski et al., 2010), cells were stimulated with different oxygen (O_2) concentrations in a hypoxic incubator (20%, 15%, 10%, 5% and 1%) over a period of 6 h because this time point yielded the highest STAR, phospho-(P)-STAR, and progesterone (P4) output in KK1 cells upon stimulation with dbcAMP. The conditioned medium was collected and stored at –20 °C for subsequent radioimmunoassay (RIA). Progesterone concentrations were assayed as previously described (Hoffmann et al., 1973).

For luciferase assays, KK1 cells were seeded in 6-well plates at a density of 5×10^5 cells per well, 24 h before transfection. FuGENE HD-transfection reagent (Roche Diagnostics Schweiz AG, Rotkreuz, CH) was used as previously described (Kowalewski et al., 2009, 2013) in 1.5 ml serum-containing medium, at a ratio of 1 μg DNA to 3.5 μl reagent in mixtures that were preincubated for at least 25 min in 100 μl serum-free media. The cells in each well were transfected with 1 μg DNA of the -151/-1 bp fragment of the murine *Star*-promoter cloned upstream of the *Firefly* luciferase reporter gene in the pGL3 basic vector (Promega, Dübendorf, CH) as previously reported (Manna et al., 2002). As shown in the deletion and site-directed mutagenesis studies in several species, this fragment of the *Star*-promoter bears the levels of the cAMP-dependent activity equivalent with the full-length promoter (reviewed in Manna et al., 2003). For normalization of the transfection efficiency, cells were cotransfected with 20 ng per well of pRL-SV40 plasmid (Promega) constitutively expressing *Renilla* luciferase driven by CMV immediate-early enhancer and promoter. Twenty-four hours after transfections, cells were used for further experiments. The activity of the promoter construct was determined after 6 h of stimulations. Luciferase activity in cell lysates was assessed by using the Dual-Luciferase Reporter System according to the protocol of the manufacturer (Promega). Briefly, cells were washed with ice-cold PBS and lysed with 300 μl Passive Lysis Buffer. Luminescence was measured in a MLX Microplate Luminometer (Dynex Technologies, GmbH, Denkendorf, Germany). The relative light units (RLU) reflect the ratio of *Firefly* luciferase luminescence to that of *Renilla* luciferase.

Transfection with HIF1α small interfering RNAs (siRNAs) under optimized conditions was done following the previously described protocol (Dyson et al., 2008). X-treme GENE siRNA transfection reagent (Roche Diagnostics) was used according to the manufacturer's directions. Silencer negative control and HIF1α siRNAs were obtained as annealed oligos from Applied Biosystems, Foster City, CA, USA. The following sequences for HIF1α correspond to the sense strand sequence: no. 1: CAG CUG ACC AGU UAC GAU UTT, and no. 2: CCU UUA CCU UCA UCG GAA ATT. They were used in an equal volume mixture at a final total concentration of 100 nM siRNAs.

2.2. RNA isolation and semi-quantitative real time (TaqMan) PCR

TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) was used for total RNA isolation following the manufacturer's directions. The semi-quantitative (TaqMan) PCR was performed in an automated fluorometer ABI PRISM® 7500 Sequence Detection System as described previously (Kowalewski et al., 2006, 2009). Briefly, total RNA was DNase-treated with DNase I recombinant, RNase-free (Roche Diagnostics) for the elimination of genomic DNA contamination. Reverse transcription utilizing random hexamers as primers was performed with reagents obtained from Applied Biosystems; 100 ng of DNase-treated total RNA were used for each sample. The following primers and 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) labeled TaqMan

probes purchased from Microsynth, Balgach, CH were used: *Star* (forward): 5′-CCG GGT GGA TGG GTC AA-3′, *Star* (reverse): 5′-CAC CTC TCC CTG CTG GAT GTA-3′, *Star* (TaqMan probe): 5′-CGA CGT CGG AGC TCT CTG CTT GG-3′, *Gapdh* (forward): 5′-GCA GTG GCA AAG TGG AGA TTG-3′, *Gapdh* (reverse): 5′-GTG AGT GGA GTC ATA CTG GAA CAT G-3′, *Gapdh* (TaqMan probe): 5′-TCA ACG ACC CCT TCA TTG ACC TC-3′. A commercially available gene-specific TaqMan® probe and primer sets of mouse-specific *Hif1α* (TaqMan system) were purchased from Applied Biosystems (Prod. No. Mm00468869_m1). All samples were analyzed in duplicates using Fast Start Universal Probe Master (ROX)® from Roche Diagnostics. For negative controls, autoclaved water instead of cDNA and the so-called 'minus RT controls' (samples that were DNase-treated but not reverse transcribed) were included. Selected PCR products were commercially sequenced (Microsynth) confirming their specificity. The comparative Ct method (ΔΔCt method), following the instructions of the ABI PRISM® 7500 fluorometer and as previously described (Gram et al., 2013; Kowalewski et al., 2006, 2009), was applied for the relative quantification of *Star* gene expression relative to the reference gene (*Gapdh*) and normalized to the calibrator (*i.e.*, the sample with the lowest amounts of the target gene transcripts). Results of the RealTime PCR are presented as *n*-fold-changes in gene expression by the hypoxic cell cultures compared with the normoxic controls.

2.3. Western blot

Whole cell lysates were prepared using NET-2 lysis buffer (50 mM Tris–HCl, PH 7.4, 300 mM NaCl, 0.05% NP-40) containing 10 ml/ml protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH, Buchs, CH) and the immunoblotting studies were performed as previously described (Gram et al., 2013). Protein homogenates (20–30 μg of total protein) were separated either on 8% or 10% polyacrylamide gel (Bio-Rad Laboratories GmbH, Munich, Germany), transferred onto methanol-activated polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and probed with specific antibodies directed against: STAR (polyclonal rabbit, a gift from Dr. DM Stocco, Texas Tech University Health Sciences Center, Lubbock, TX, USA (Clark et al., 1994), used at dilution 1:5000), 3βHSD (polyclonal rabbit, a gift from Dr. JI Mason, University of Edinburgh, UK (Lorence et al., 1990), used at dilution 1:2000), P450scc (rabbit polyclonal, AB12491, Cell Signaling Technology, Inc., Danvers, MA, USA, dilution 1:1000), and HIF1α (polyclonal rabbit, AB3716, Cell Signaling Technology, dilution 1:1000). For loading controls and semi-quantitation of target protein expression, PVDF membranes were reblotted with either mouse monoclonal antibody against GAPDH (sc-47724) or mouse monoclonal against βACTIN (sc-81178), both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, used at dilutions 1:1000. Secondary antibodies were: donkey anti-rabbit HRP-labeled secondary IgG from Pierce Biotechnology, Rockford, IL, USA, and anti-mouse IgG conjugated to HRP from Promega, both used at dilutions 1:30'000. Signals were detected with SuperSignal West chemiluminescent substrate (Pierce Biotechnology) according to the manufacturer's protocol and visualized with ChemiDoc XRS+ System and Image Lab Software, in the presence of the Precision Plus Protein Standard (molecular weight marker) ranging from 10 to 250 kDa (all from Bio-Rad). A fine-point phosphorescent ink marking pen Glow Writer (MIDSCI, St. Louis, MO, USA) was used to annotate the visible protein ladder on membranes prior to Western blot detection with ChemiDoc XRS+ System. An exemplary resolution of the standard is presented in Fig. 1. The optical density of bands was assessed using ImageJ software.

2.4. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay kit obtained from Merck KGaA, Darmstadt, Germany was used according to the directions of the manufacturer and as previously described (Manna and Stocco, 2007).

Antibody specific to HIF1α was the same as for Western blotting and rabbit IgG was used for the negative control. The subsequent PCR reactions with the recovered DNA (100 ng for each sample) after the immunoprecipitation procedure, as well as with the DNA isolated from the input homogenates, was performed with specific primers, forward: 5′-CTG GTC CTC CCT TTA CAC AGT C-3′, and reverse: 5′-GGC GCA GAT CCA GTG GGC TGC-3′, amplifying the -170/-1 fragment of the proximal murine *Star*-promoter. PCR products were visualized on 2% agarose gel prestained with ethidium bromide. Semi-quantitation of the ChIP results was done by determining the optical density of bands assessed by ImageJ software.

2.5. Statistical analysis

All experiments were repeated at least three times each. Representative Western blots are shown for each experiment. Luciferase assays and Real Time (TaqMan) PCR were performed in duplicates. Statistical analysis was done by either one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, or unpaired two-tailed Student's *t*-test, with the statistic software program GraphPad 3.06 (GraphPad Software, San Diego, CA, USA). Results are presented as the mean ± standard deviation, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Validation of hypoxic experimental conditions. Effects of hypoxia on STAR, P450ssc, 3βHSD expression and steroid production in KK1 granulosa cells

For all experiments, hypoxia was induced by lowering the oxygen (O_2) concentration in cell cultures. The potential of KK1 granulosa cells to respond to the hypoxic stimuli was determined by analyzing HIF1α expression by Western blots. A representative result is presented in Fig. 1, showing a significant increase (ANOVA $P < 0.0001$) in HIF1α protein levels in cells cultured under hypoxic conditions for 6 h. Since both the expression of βACTIN and GAPDH remained unaffected by hypoxia in our cell culture model, they were used for internal loading controls in experiments requiring the semi-quantitation of protein expression (Figs. 1, 4, 5 and 6).

Next, cells were incubated in serum-free DMEM/F12 medium with progressively decreased O_2 concentrations (20%, 15%, 10%, 5% or 1%) in the presence of increasing concentrations of dbcAMP. The conditioned culture medium was then subjected to progesterone (P4) measurement by RIA, revealing strongly significant effects of treatments on steroid production (ANOVA $P < 0.0001$) (Fig. 2A). Whereas, as expected there was a dose-dependent ($P < 0.01$) increase of P4 production observed in cells treated with dbcAMP under normoxia, both, the basal and dbcAMP-stimulated P4 production was decreased in cells incubated under severe hypoxia compared with the normoxic control in every one of the treatment groups (with or without dbcAMP). In the control group (no dbcAMP) P4 was reduced only at 1% O_2, while in the 0.1 mM dbcAMP and 0.3mM dbcAMP treated groups both 1% and 5% O_2 reduced P4 production. The opposite effect was, however, observed following culture under partially reduced (10%) O_2 concentration in cells treated with 0.1 mM dbcAMP, which exhibited significantly increased P4 output up to 59% (1.6-fold, $P < 0.01$) under this lower PKA background. When cells were stimulated with 0.3 mM dbcAMP the maximal P4 production was not further increased ($P > 0.05$) by partial hypoxia. Hence, partial hypoxia appeared to shift the steroid production in cultured granulosa cells to the left.

In addition to the expected increase of *Star* mRNA in response to dbcAMP under normoxia (Fig. 2B), partial hypoxia (10% O_2) by itself significantly ($P < 0.05$) induced the expression of *Star* mRNA and this effect was potentiated when dbcAMP was added to the

treatment medium ($P < 0.01$ at 0.1 mM dbcAMP). Accordingly, when the proximal -151/-1 bp *Star* promoter construct was transfected into cells, its activity was upregulated ($P < 0.05$) under partial hypoxia (10% O_2) (Fig. 3). Pre-treatment of cells for 30 min with a specific inhibitor of the PKA pathway, H89 (20 μM; see Kowalewski et al., 2010; Manna et al., 2006, 2007, 2009, 2014) (Sigma-Aldrich Chemie GmbH) prevented this increase (Fig. 3).

Western blot analysis clearly demonstrated the expected dose-dependent increase ($P < 0.01$) of STAR protein expression by cells following treatment with dbcAMP compared with control cells (Fig. 4). The STAR levels were strongly elevated ($P < 0.01$) in cells cultured under 10% O_2 but this effect ceased when O_2 was decreased to 5% (Fig. 4). While it had a positive impact on STAR protein expression, partial hypoxia (10% O_2) did not alter expression of the steroidogenic enzymes 3βHSD and P450scc, which also remained unaffected by dbcAMP (Fig. 4) under normoxic conditions.

3.2. Effects of hypoxia on STAR expression under hypoxia are HIF1α-mediated

Acting as a transcription factor, HIF1α binds to the specific recognition sites of target genes and induces their expression. Thus, having observed the hypoxia-mediated increase of STAR expression, it seemed possible that HIF1α may be involved in this process. The control and dbcAMP-treated cells were incubated under partial hypoxia (10% O_2) with or without echinomycin (5 nM; Cayman Chemical Inc., Ann Arbor, MI, USA), an inhibitor of HIF1α-mediated gene transcription. Echinomycin prevented the dbcAMP-stimulated increase of STAR protein expression, both in normoxic and hypoxic environments (Fig. 5). This effect was even more strongly manifested when the expression of HIF1α was suppressed by siRNA interference. Thus, following the suppression of HIF1α expression (Fig. 6A), cells transfected with siRNA significantly ($P < 0.05$) reduced

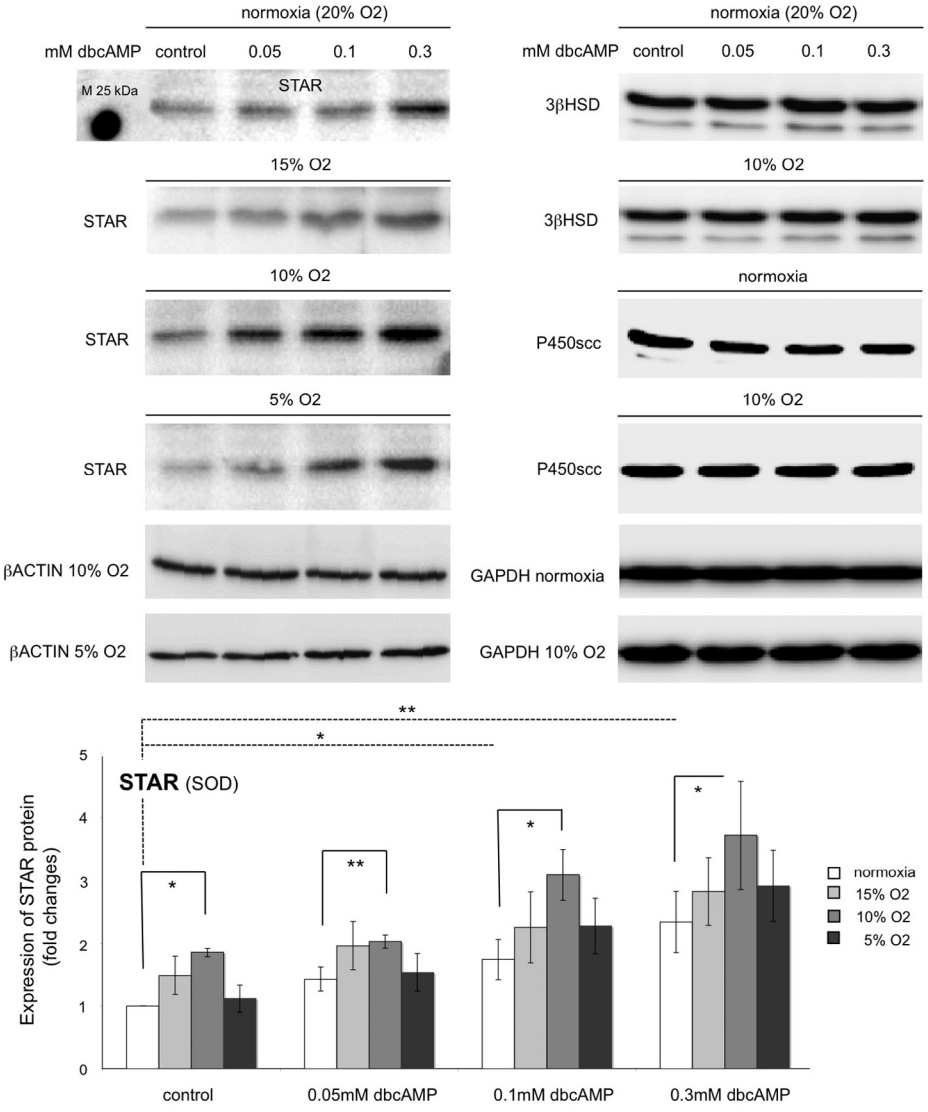

Fig. 4. Effects of hypoxia on STAR protein expression in KK1 mouse granulosa cells. Cells were cultured for 6 h in DMEM/F12 medium in the presence of increasing dbcAMP content at different O_2 concentrations. Following stimulation, cells were collected and homogenized, and 20 μg of the lysate was used for Western blot analysis of STAR (30 kDa), 3βHSD (42 kDa), P450scc (45 kDa), GAPDH (37 kDa) and βACTIN (45 kDa). The average standardized optical density (SOD) for STAR is shown. One-way ANOVA ($P < 0.0001$ for control, $P < 0.0081$ for 0.05 mM dbcAMP, $P < 0.006$ for 0.1 mM dbcAMP and $P < 0.011$ for 0.3 mM dbcAMP-stimulated cells), followed by Dunnett's multiple comparison test was applied; all samples were compared against the normoxic control in each group. (*) indicates $P < 0.01$ and (**) indicates $P < 0.05$. Additionally, one-way ANOVA followed by Dunnett's Multiple Comparison Test was applied for all controls (dashed lines indicate: (*) $P < 0.05$, (**) $P < 0.01$). Representative Western blots are shown. An exemplary 25 kDa molecular weight marker of the Precision Plus Protein Standard is presented.

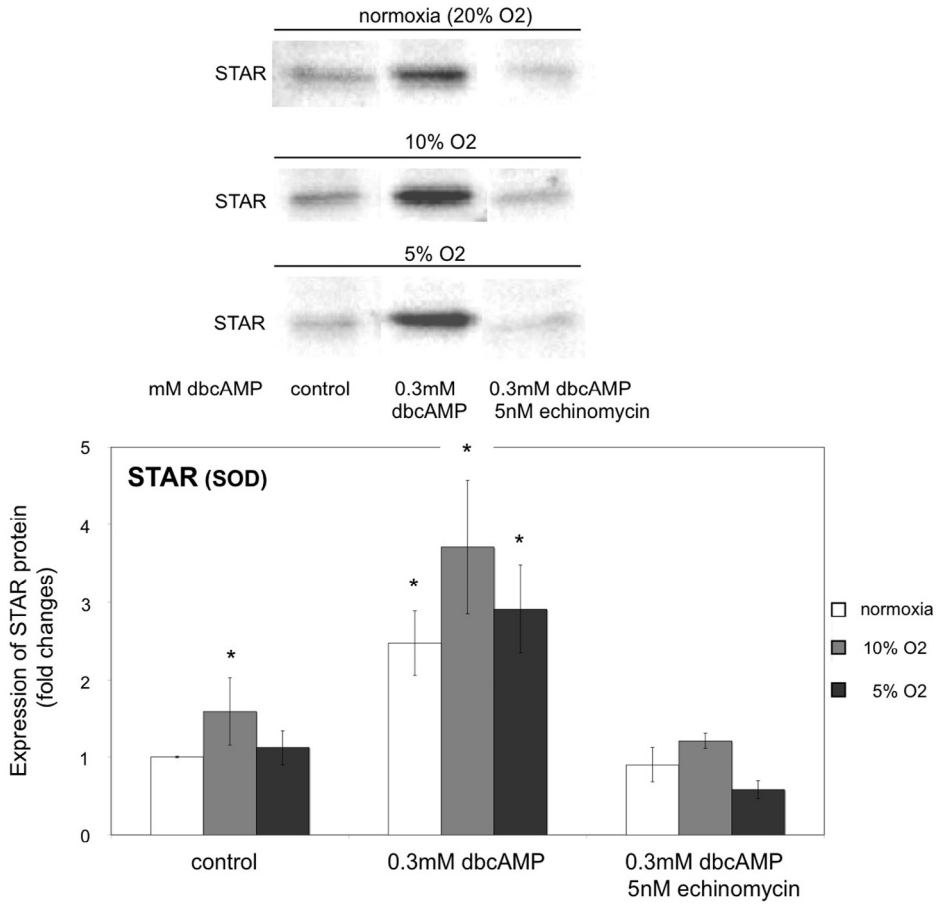

Fig. 5. Blocking of HIF1α transcriptional activity prevents hypoxia-mediated STAR expression. Cells were treated in DMEM/F12 for 6 h in the presence or absence of echinomycin (5 nM). Following stimulation, cells were collected and homogenized; cell lysate was used for Western blot analysis. The average standardized optical density (SOD) for STAR (30 kDa) is shown. Representative Western blots are presented. One-way ANOVA with $P < 0.0001$ followed by Dunnett's multiple comparison test was applied; all samples were compared with the untreated normoxic control; (*) indicates $P < 0.01$.

the basal and stimulated STAR mRNA and protein expression compared with the respective negative control siRNA (Fig. 6B and C).

To determine whether HIF1α can directly bind to *Star* promoter, ChIP studies were performed. Cross-linked, sheared chromatin from control and dbcAMP-treated KK1 cells cultured under normoxia and partial hypoxia (10% O_2) was immunoprecipitated with anti-HIF1α antibody. The recovered DNA was subsequently subjected to semi-quantitative PCR with primers specific for the proximal *Star* promoter region. As presented in Fig. 7, 170 bp amplicons were detected with DNA derived from all samples, indicating an involvement of HIF1α in basal and dbcAMP- and/or hypoxia-stimulated *Star* gene transcription. The association of HIF1α with the promoter was potentiated under reduced O_2 compared to the normoxic control ($P < 0.003$ and $P < 0.001$, in cells incubated with and without dbcAMP, respectively) (Fig. 7).

4. Discussion

The present study was based on the observation of apparent discrepancies between the morphological characteristics of growing ovarian follicles, revealing a restricted vascular supply resulting in a physiologically hypoxic follicular milieu, and the negative effects of hypoxia on steroidogenesis and/or STAR expression postulated by some authors (Jiang et al., 2011; Nishimura et al., 2006). Therefore, the influence of progressively reduced O_2 levels on STAR protein expression and on steroidogenesis was investigated, using murine KK1 granulosa cells as an experimental model. The main focus was on the potential role of HIF1α in these processes since it is a prominent marker and a major regulatory factor involved in cellular responses to hypoxia.

It is noteworthy that in the available literature $CoCl_2$ is frequently described as an agent mimicking hypoxia and therefore it is often used in studies investigating this condition. In fact, however, its function is mostly in stabilizing HIF1α and generating excessive intracellular accumulation of this factor and, thus, $CoCl_2$ only to some extent and indirectly leads to hypoxia-like conditions. Therefore, taking into account the great complexity of hypoxia, the use of $CoCl_2$ was considered inappropriate in our studies for investigating the impact of hypoxia on steroidogenic properties of granulosa cells. Instead, decreasing O_2 concentrations (20%, 15%, 10%, 5%, 1%) were applied and hypoxia was efficiently induced, as indicated by concomitantly rising cellular HIF1α expression.

Whereas expectedly, under very low O_2 (5% and 1%) cells decreased their steroidogenic activity, an opposite effect was observed when the O_2 concentration was only partially decreased to 10%. Thus, it was interesting that in cells incubated at 10% O_2 steroid levels were strongly elevated with a lower background of PKA activity, *i.e.*, when 0.1 mM dbcAMP was added to the treatment medium. This increase was similar to the maximal P4 output observed in normoxic cells stimulated with 0.3 mM dbcAMP. Furthermore, the partial hypoxia resulted in strongly enhanced STAR mRNA and protein expression, without having an impact on the expression of the steroidogenic enzymes 3βHSD and P450scc.

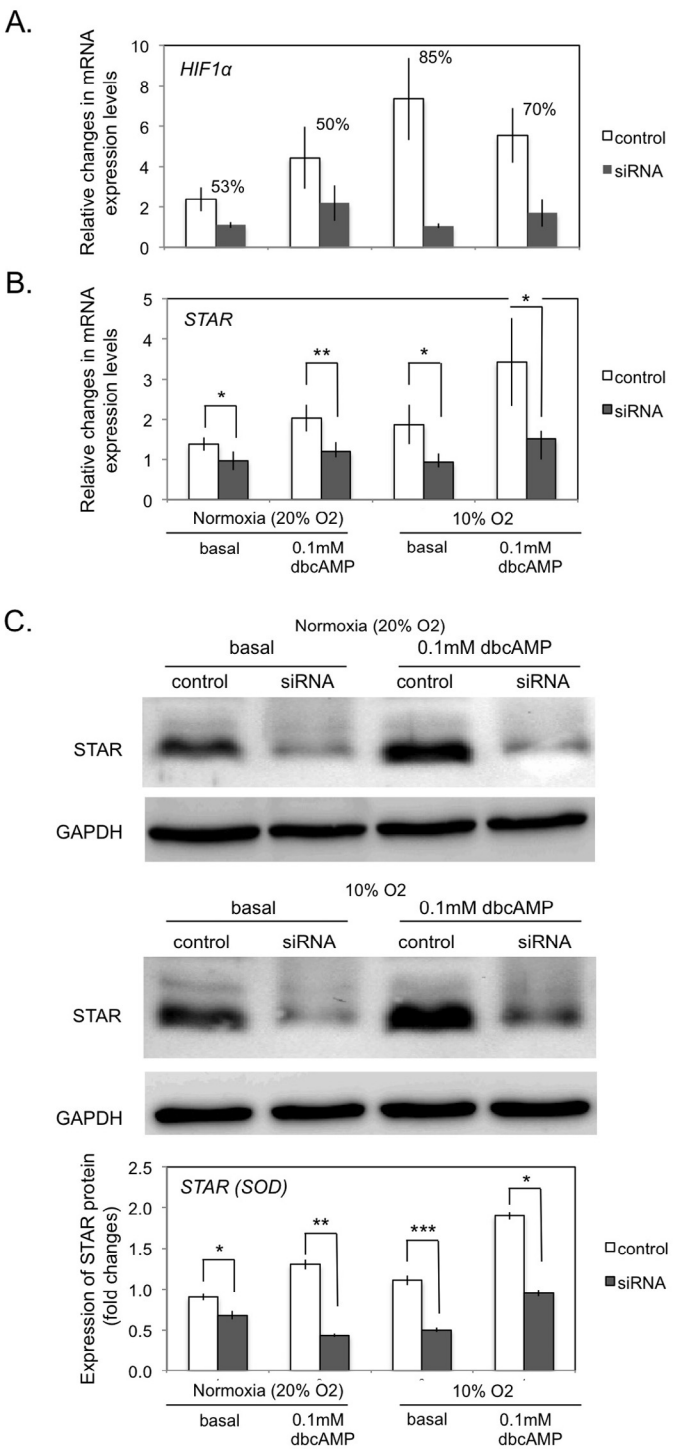

Fig. 6. Suppression of HIF1α expression decreases basal and stimulated STAR expression under normoxia and partial hypoxia (10% O_2). Cells were transfected with either a silencer negative control or HIF1α siRNA as described in Materials and Methods. After 48 h of transfection, cells were treated without (basal) or with 0.1 mM dbcAMP, either under normoxic conditions or partial (10% O_2) hypoxia for an additional 6 h. (A) *Hif1α* expression was determined by Real Time (TaqMan) PCR showing strong decrease (up to 85%) of mRNA levels in siRNA transfected cells. (B) *Star* mRNA expression was determined by Real Time (TaqMan) PCR. (C) Harvested cells were subjected to cellular protein preparation for Western blot analysis. Representative Western blots show the expression of STAR (30 kDa) and GAPDH (37 kDa). Semi-quantitative analysis was performed; the average standardized optical density (SOD) for STAR is shown. An unpaired two-tailed Student's *t*-test was applied in B and C. Bars with (*) differ at $P < 0.05$, bars with (**) differ at $P < 0.01$ and bars with (***) differ at $P < 0.03$.

Blocking HIF1α transcriptional activity with echinomycin prevented the hypoxia-mediated increase in STAR expression. Since it could be considered that echinomycin would inhibit functions of other genes by interfering with the DNA-binding activity of their transcription factors, we performed gene-targeted knockdown of HIF1α expression, observing the same result as with echinomycin, thus further implicating this gene in regulating STAR expression. An interesting finding in our current study is the observation that HIF1α regulates not only the dbcAMP- and/or hypoxia-stimulated STAR expression, but also the basal expression level. This was clearly evident in HIF1α knockdown experiments. These findings cumulatively corroborate the results reported by Tam et al. (2010) who showed an increase in HIF1α expression in hCG-stimulated granulosa cells in growing ovarian follicles.

Clearly, the hypoxia-stimulated expression of STAR was PKA-dependent as shown in the experiments utilizing the PKA blocker H89. Consequently, since the increased expression of STAR appears to result from upregulated HIF1α expression, it is possible that STAR could be sufficiently activated, *i.e.*, phosphorylated, even in the presence of a lower PKA background, resulting in the increased progesterone output. Following further decreases of O_2 concentration, negative effects on STAR expression and cellular steroidogenic activities were observed. Since the PKA pathway is the major signaling route regulating STAR expression and function (Manna et al., 2003), consistent with the expected dose-dependent increase in P4 output in cells treated with dbcAMP as shown in Fig. 2, these negative effects occurring under severe hypoxia may result from strongly reduced PKA activity, as described previously (Jiang et al., 2011). This also corroborates the decreased steroidogenic activity observed in cells under severe hypoxia.

The results from Real Time PCR and the promoter data indicate that incubation of cells under hypoxic conditions induced an increase in *Star* promoter activity, suggesting that HIF1α-mediated STAR expression may be regulated at the transcriptional level. Thus, we felt prompted to investigate if HIF1α would directly regulate *Star* expression by binding to its promoter. Consequently, the ChIP assay was performed and the binding of HIF1α to proximal murine *Star* promoter was shown for the first time. Interestingly, HIF1α was associated with the *Star* promoter under basal conditions in unstimulated granulosa cells and this binding was further increased following the upregulation of HIF1α expression under partial hypoxia.

We next searched for presence of the hypoxia responsive element (HRE) consensus sequence within the murine proximal *Star* promoter. A 5′-CAAGTG-3′ sequence closely resembling the 5′-RCGTG-3′ HRE consensus sequence with only one mismatch was located at -55/-60 bp upstream of the transcription initiation site. This fragment is identical to the fully functional HIF1α binding site identified in the rat IGFB1 gene described by Scharf et al. (2005). Mutation of this sequence within the rat IGFB1 promoter luciferase construct abolished induction by hypoxic conditions, in that study. The functional relevance of this putative HIF1α recognition site, and its possible interaction with other transcription factors regulating *Star* promoter expression is worth attention and thus warrants further investigations.

Although there is no strict demarcation between physiological and extreme hypoxia, the complex functional relationship between hypoxia and its role in steroidogenesis probably needs to be seen as a balance between the beneficial transcriptional activity of HIF1α on *Star* promoter and the negative effects of hypoxia. The latter, diminishing, *e.g.* PKA activity may result in apoptosis or even necrosis (Piret et al., 2002). In the experimental model used in our study, this balance seemed to be reached at the 10% O_2 concentration, constituting a threshold value between the positive and adverse effects of hypoxia. Surely, the functional physiological implications of this finding require further elucidation, with, *e.g.*,

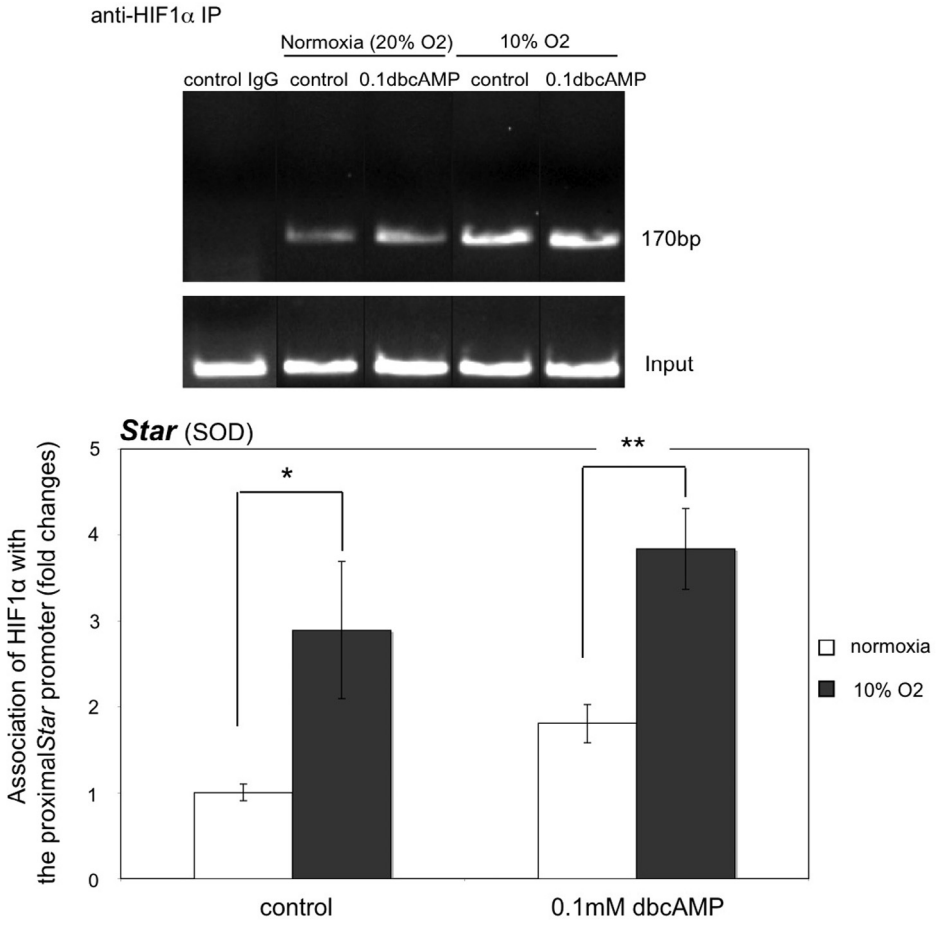

Fig. 7. Association of HIF1α with the murine proximal *Star* promoter. Cells were treated for 6 h in DMEM/F12 medium without (control) or with 0.1 mM dbcAMP, either at normoxia (20% O_2) or under partial hypoxia (10% O_2). ChIP assay was performed as described in Materials and Methods. Cross-linked, sheared chromatin was subjected to immunoprecipitation (IP) either with irrelevant IgG (negative control) or anti-HIF1α antibody. Recovered DNA was used for PCR using primers amplifying the -170/-1 bp region of murine *Star* promoter. Representative amplicons are presented. Semi-quantitative analysis was performed; the average standardized optical density (SOD) for *Star* is shown. An unpaired two-tailed Student's *t*-test was applied. Bars with (*) differ at $P < 0.01$, and bars with (**) differ at $P < 0.002$.

experiments involving primary cells. It seems, however, to fit well with the aforementioned critical role of HIF1α during follicle growth and maturation and ovulation (Tam et al., 2010).

In conclusion, the data obtained in the present study provide the first evidence that HIF1α is actively involved in direct regulation of basal and stimulated STAR protein expression, which under partial hypoxia is capable of increasing the steroidogenic capacity of granulosa cells. Since the effects of HIF1α may be influenced by other factors, *e.g.*, those regulating its intracellular stability or transcriptional activity, and interaction with other transcription factors, aspects regarding the exact mechanism by which it actually performs its role in regulating STAR expression and steroidogenesis await further investigation.

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